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A Novel Strategy to control *Candida* Biofilms

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ABSTRACT:
The limitation of antifungal drugs in preventing *Candida albicans* biofilm formation on silicone rubber voice prosthesis in laryngectomised patients has been well documented. This study was designed to assess a novel approach to control the colonization by yeasts. *C.albicans* biofilms were exposed to commercially available probiotics alone and in combination with Amphotericin B. The reduction in biofilm formation was verified by viable count technique. Exopolysaccharide formation, indicative of the amount of biofilm formed, was determined by Anthrone method. Biofilms formed on glass slides were observed by Allison and Sutherland staining. The minimum inhibitory concentration of Amphotericin B for *C. albicans* was 2.5 microgram per milliliter which was lower than the minimum bactericidal concentration. All the probiotic preparations when used in combination with Amphotericin B caused reduction in the viable count and exopolysaccharide formation by *Candida*. These reductions observed were more than those induced by probiotics or Amphotericin B separately. Probiotic preparation 3 in combination with Amphotericin B could cause complete elimination of biofilm and exopolysaccharide formation on silicone rubber. Thus probiotics could represent an important adjunct to conventional antifungal therapy. We believe this is the first study to demonstrate the use of commercially available probiotics in combination with an antifungal drug as a mode of therapeutic intervention for preventing *Candida* biofilms.

Key words: Probiotics, *Candida albicans*, Biofilms, Amphotericin B, silicone rubber.

INTRODUCTION

The main cause of malfunctioning of the silicone rubber voice prosthesis in laryngectomised patients is its colonization by bacterial [1] and fungal biofilms.[2] Once a *Candida* biofilm forms, it exhibits high resistance to antimycotics, remaining a reservoir of infection until the implant is removed[3]. Also the ingrowth of yeasts into the silicone rubber offers a protective environment, making complete elimination of the biofilm impossible. Thus an indwelling voice prosthesis usually has to be replaced every 3 to 4 months.

Though the consumption of probiotics is largely motivated by health claims related to the urogenital and lower digestive tract, a previous investigation by Van der Mei et al has demonstrated the potential benefit of using some isolated single strains of probiotic bacteria for controlling yeasts in oropharyngeal biofilms.[4-6]. Lactobacilli can interfere with the colonization of yeasts by production of hydrogen peroxide and lactic acid, competitive addition, displacement and the release of anti-adhesive bio-surfactant[7,8].

Despite evidence to suggest that certain individual strains of probiotic cultures could cause significant reduction of *Candida* biofilm formation, no further research has been done to check the usefulness of commercially available probiotic preparations, for the same purpose. With probiotics there are strain to strain differences and it has been seen that different probiotic species and strains confer different health benefits. In this study we tested the hypothesis that certain specific probiotic strain combinations would be more effective in reducing *Candida albicans* biofilm formation than others.

We selected five currently available commercial probiotic preparations (containing different combinations of probiotic cultures) commonly prescribed by physicians in India and have investigated them alone as well as in combination with the antifungal drug, Amphotericin B for reducing yeast biofilm. Thus in our present study, we have explored the use of a novel strategy for controlling the prevalence of *Candida albicans* in a biofilm.

MATERIALS AND METHODS

Culture & Growth conditions

Candida albicans culture obtained from a local hospital was grown overnight at 37^{o} C in Yeast Peptone Dextrose broth supplemented with 50 mM sucrose to obtain cells in budding phase. The cells were harvested, washed in sterile phosphate buffer saline and standardized to an O.D. of 0.6 at 530nm. (ErmaInc. Colorimeter). The commercially available probiotics investigated in this study were labeled as preparation 1, 2, 3, 4 and 5 (See Table 1).

Table1: The composition of commercially available probiotics investigated in this study

Probiotic Preparation no.	Composition of the probiotic preparation	Brand name	Manufacturing company
1	*Lactobacillus acidophilus* 2000L +Ca pantothenate +Thiamine+Niacinamide + Vit. B12 + Vit.B6	Becelac	Dr.Reddy's Laboratories, India
2	*Streptococcous faecalis T110*- 30 million + *Closridium butyricum* 2million + *Bacillus* mesentricus 1million + *Lactobacillus sporogenes*	Bifilac	TIL Pharma Div. India
3	*Bifidobacterium Longum* +*Lactobacillus rhamnosus* +*Lactobacillus acidophilus* +*Saccharomyces boulardii*	Darolac	Aristo Pharma Pvt Ltd. India
4	*Lactobacillus lactis* 490 million + *Lactobacillus acidophilus* 490 million +*Streptococcus thermophilus* 10 million +*Streptococcus lactis* 10million	Lactisyn	Griffon Pvt. Ltd India
5	*Lactobacillus* species	Vyzylac	Unichem laboratories Ltd,India

Determination of minimum inhibitory concentration of Amphotericin B against C. albicans

Using stock solution of Amphotericin B (5ug/ml) various dilutions was prepared. 0.1 ml of the 48 hr old *C.albicans* culture was added to all the tubes. Positive and negative controls were maintained. The tubes were incubated at 37^{o}C for 48 hrs and the lowest concentration that did not show growth corresponded to the minimum inhibitory concentration.

Determination of minimum bactericidal concentration of Amphotericin B against C. albicans

0.1 ml of culture from the tube indicating minimum inhibitory concentration and all higher concentrations beyond it,were surface spread on Yeast Peptone Dextrose agar and incubated at 37^{o}C for 48 hrs. The minimum concentration showing no growth corresponded to the minimum bactericidal concentration.

Preparation of silicone rubber sections & biofilm formation

2cm×2cm sections of silicone rubber (Silicone Rubber Industry, India) were rinsed thoroughly in warm tap water followed by washing using methanol and sterile distilled water. The silicone rubber sections were treated with detergent for 24hrs. The sections were then thoroughly rinsed with distilled water & exposed to UV light for 1hr prior to use. Sterile silicone sections were placed in coplin jars with 40ml of YPD medium with 50mM sucrose. 1ml of 0.6 O.D.culture of *C. albicans* was added to the medium and incubated for 72hrs at 37 ^{0}C for biofilm formation.

Susceptibility of biofilm to probiotic cultures

The silicone rubber sections with 72 hr old biofilm were placed in fresh Brain heart infusion medium (BHI) with 0.5% lactose and exposed to probiotic preparations for 5 minutes thrice a day for two days. The incubation temperature was maintained at 37^{0} C.

Susceptibility of biofilm with probiotic cultures along with Amphotericin B

Each silicone rubber section was placed in fresh BHI medium (with 0.5% lactose) with sublethal concentration of Amphotericin B (0.95ug/ml) for two days during which they were exposed to probiotic preparations for 5 minutes thrice a day and incubated at 37 degree C.

Control

The silicone rubber sections with 72 hr old biofilm were placed in fresh BHI medium (with 0.5% lactose) without Amphotericin B and incubated at 37 degree C for 48 hrs.

Evaluation of biofilm

Each silicone rubber section was dip-washed in sterile PBS and gently swirled to remove any adherent medium. The sections were then placed in 2 ml PBS and the adherent cells harvested by scraping with sterile scalpel. Suspension was homogenized,1ml was removed and used for viable count and the remaining suspension retained for polysaccharide analysis. For determining viable count, the homogenized suspension was serially diluted with PBS, 0.1ml of the culture was spread on Saboraud's agar plates and incubated at 37 ^{0}C for 48 hrs. To extract EPS, 4ml of PBS (pH11) was

added to 1ml of the homogenized suspension and kept at 80 degree C for 1 hr. This suspension was centrifuged twice at 3000rpm for 40 minutes and the supernatant used for polysaccharide analysis by Anthrone method (9). The concentration of glucan was determined by measuring absorbance at 620nm.

Allison and Sutherland staining

Candida biofilms similarly formed and treated on glass slides were covered with 10mM cetyl pyridinium chloride and air dried for 20 minutes .The heat fixed slides were stained for 15 minutes with Congo red and 10%v/v Tween 80, washed with water, air dried and observed.

Experiments were performed in triplicate and repeated three times. The data was then statistically analysed using student's t-test. Values were considered significant when p value was less than 0.05.

RESULTS

The MIC and MBC of Amphotericin B for *C. albicans* were found to be 2.5 ug/ml and 3.8 ug/ml respectively. The mean values of viable count and % reduction in EPS content of the biofilm are mentioned in Table 2.

These results show that the exposure to sublethal concentration of Amphotericin B along with probiotic cultures, decreases the viable count by 10 to 100 fold. Probiotic preparation 3 in combination with Amphotericin B could completely prevent the formation of *C. albicans* biofilm.

On performing Allison and Sutherland staining, the direct microscopic examination of the control biofilm slide showed compactly packed mature cells with intense red coloured EPS around them. (Figure 1)

Biofilm exposed to sublethal concentration of Amphotericin B showed scattered biofilm formation but stained with the same intensity as the stained EPS in control biofilm. Biofilm exposed to only Probiotic preparation 3 showed clumps of cells with some EPS in between the cells, whereas exposure to the same preparation but in combination with Amphotericin B showed complete destruction of *Candida* biofilm.

Biofilm exposed to sublethal concentration of Amphotericin B showed glucan content of 0.36mg which was less than glucan content of a 72hr old control biofilm (1.26mg). Biofilm exposed to Probiotic preparation 1, 2, 3, 4 and 5 showed a polysaccharide content of 0.42mg, 0.87mg, 0.18mg, 0.26mg and 0.3 mg respectively. When the biofilms were treated with sublethal concentration of Amphotericin B along with intermittent exposure to probiotic preparations1, 2, 4 and 5, a polysaccharide content of 0.12mg, 0.14 mg, 0.07mg ,0.1mg was obtained respectively. Glucan content of biofilm exposed to probiotic preparation 3 along with the Amphotericin B was found to be nil. Decrease in polysaccharide concentration was expressed as percentage reduction compared to the control biofilm (which was taken as 100%).

DISCUSSION

Candida albicans is a yeast that constitutes the normal flora of the oral cavity. Disequilibrium of the microbiota in patients favours the growth of these opportunistic microorganisms and their increased presence is common in cancer patients receiving chemotherapy or radiotherapy. The exopolysaccharide matrix secreted by *Candida* is of primary importance in its colonization [10]. Following radiotherapy there is disruption of healthy oral microflora due to reduced salivary flow resulting in increased susceptibility to *Candida* infections. It has been observed that the colonization by *Candida* on the indwelling voice prosthesis in laryngectomized cancer patients leads to its deterioration.

Investigations to prevent *Candida* biofilm formation have focused on the use of various antimycotics [11].The limitation of these drugs in preventing *Candida* biofilm formation on silicone rubber voice prosthesis in laryngectomised patients has been well documented. A slow release tablet containing the antimycotic miconazole nitrate has been reported to be unsuccessful in reducing the number of yeasts in the biofilms on the valve side of prostheses[12]. Similar results have been found with the use of amphotericin B lozenges [13] and rinsing the oral cavity twice a day with nystatin suspension [14].

As an alternative, in the year 2000, Van der Mei et al used isolated single strains of probiotic cultures for killing *C. albicans* cells within a biofilm and reported some success [4]. Among the cultures which were tested, *Lactobacillus rhamnosus*, *Streptococcus thermophilus* and *Lactobacillus lactis* 53 could significantly reduce the number of yeasts in the biofilm to 50%, 33% and 4% of the control respectively.

However, this study had examined the use of pure cultures of probiotics which would be relatively inaccessible in clinical situations, thus it was worthwhile investigating whether this protection could be also be provided by commercially available probiotic preparations.

Combinations of probiotics prescribed for clinical use are often given with no reference to their specific effects.This has been challenged by certain researchers [15] who suggest that some probiotics are predominant

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for specific conditions such as certain strains like *Lactobacilli GG* can treat atopic eczema while *L.casei* was shown to boost immunity in response to respiratory infections in mice[16].

Another strategy worth exploring was the use of various combinations of probiotic cultures not only alone but also in combination with a commonly prescribed antimycotic drug for preventing Candidal biofilm formation.

Table 2: Comparative study of the effect of probiotic cultures alone and in combination with Amphotericin B on C . albicans biofilm data obtained after performing viable count of cells present within the biofilm and estimating the amount of exopolysaccharide (EPS) formed by Anthrone test.

Treated with probiotic preparation no.	**ONLY PROBIOTIC**		**PROBIOTIC & AMPHOTERICIN B**	
	CFU/BIOFILM	% reduction in EPS	CFU/BIOFILM	% reduction in EPS
1	5.6×10^{7}	67	3×10^{7}	90
2	20.8×10^{7}	32	33.2×10^{6}	89
3	3.6×10^{7}	79	4	100
4	4.8×10^{7}	76	5×10^{6}	94
5	5.4×10^{7}	85	26.8×10^{6}	92

BIOFILM	CFU/BIOFILM	% reduction in EPS
CONTROL	4.9×10^{10}	0%
Exposed to amphotericin B	10×10^{7}	71%

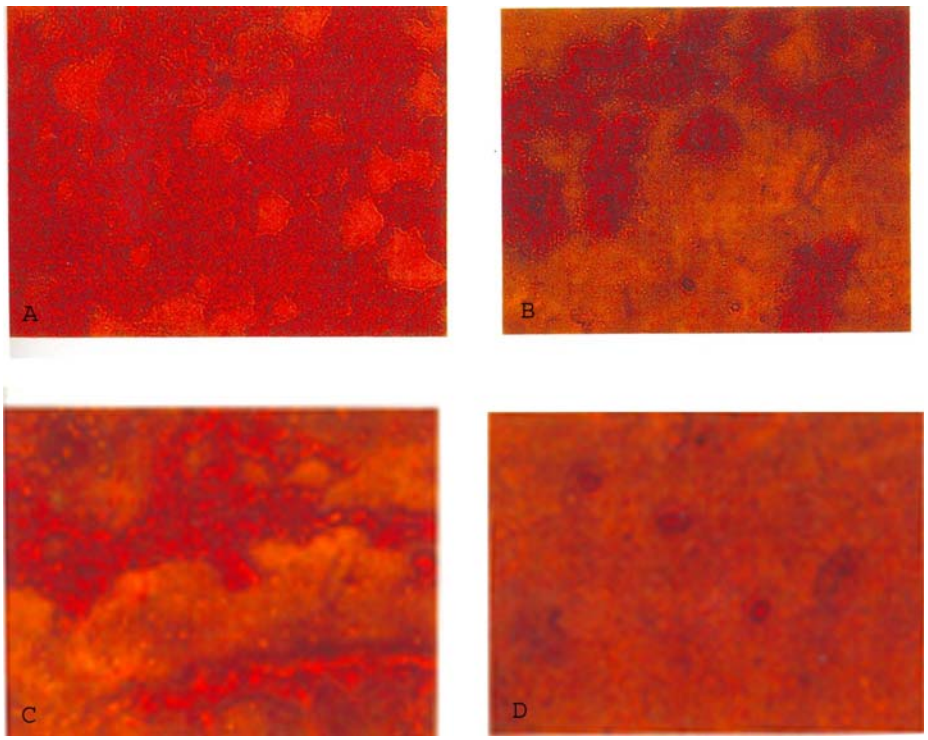

Figure1: Allison and Sutherland Staining of biofilms formed on glass slides. Magnified 1000X. (A)Control showing heavy biofilm formation (B) After exposure to probiotic preparation 1 and Amphotericin B (C) After exposure to only probiotic preparation 4 (D) After exposure to probiotic preparation3 and Amphotericin B.

In our study, we exposed the biofilms to five different commercially available probiotic preparations (containing different combinations of probiotic cultures) available in India, both alone, as well as in combination with Amphotericin B. We chose Amphotericin B as an antifungal drug of choice, since it is a narrow spectrum antifungal agent. Other antifungal agents belonging to azole group are broad spectrum and thus could interfere with the activity of probiotics. Amphotericin B, an important antimycotic agent, binds to ergosterol component in the cell membrane and leads to formation of pores in the membrane, causing leakage of cellular contents.

In order to conduct the study the ideal model system would be laryngectomized cancer patients with indwelling silicone rubber voice prosthesis. However for obvious ethical reasons such a model system was ruled out. An alternative used by several researchers is an artificial throat system consisting of silicone rubber voice prostheses, placed in a Robbin's device which is perfused with medium and culture. We have devised a novel, low cost and simple method for carrying out preliminary screening of a spectrum of probiotic strains for their potential clinical application by employing sections of silicone rubber placed in coplin jars, maintained at an ambient temperature. On the basis of our study we could identify which probiotic preparation alone as well as in combination with amphotericin B was most effective in preventing *Candida* biofilm formation on silicone rubber.In our studies, we found that the concentration of the antimycotic drug required to inhibit planktonic *Candida* cells to be less than that required to kill them [17].

In the present study we have tested a combination of probiotic cultures. Amongst the commercial preparations examined, preparation 3 (a mixture of *Bifidobacterium longum, Lactobacillus rhamnosus, Lactobacillus acidophilus and Saccharomyces boulardii.*) was found to be most effective in reducing viable count of *Candida* cells within a biofilm. This preparation in combination with Amphotericin B could cause almost complete elimination of biofilm as well as EPS formation and thus deserves more attention in further clinical studies.

It is worthwhile investigating whether this effect would be seen even with lower concentrations of Amphotericin B. This would help in formulating a drug treatment regime for patients and allow administering lower doses of the antifungal drug Amphotericin B, which is commonly associated with nephrotoxicity.

Our data indicated that amongst the probiotics assessed, Preparation 2 (consisting of a mixture of *Streptococcous faecalis, Closridium butyricum, Bacillus mesentricus* and *Lactobacillus sporogenes*) caused the least amount of reduction in viable count as well as in EPS formation by *Candida.*

With the exception of preparation 2, all the probiotics tested were found to be more effective than Amphotericin B alone.The reduction in the number of viable yeast cells was even greater when probiotics were used in combination with Amphotericin B.This could possibly be due to the biosurfactants produced by probiotics, which interfere with the adhesion of *C.albicans* or have some activity against the EPS matrix, allowing the penetration of Amphotericin B into the biofilm. Some probiotic cultures like *L. lactis53* are even known to produce antimycotics.

In conclusion, the results of our present study indicate the potential benefits of combined therapy, involving the use of probiotic bacteria in combination with Amphotericin B, for controlling the prevalence of *Candida* in biofilms. Probiotics could thus represent an important adjunct to conventional antifungal therapy for laryngectomised patients which would confer health benefits without involving any harmful side effects traditionally associated with chemotherapeutic drugs. Further invivo tests and clinical trials would validate the usefulness of this approach and lead to the introduction of a novel mode of therapeutic intervention.

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REFERENCES

[1] Neu T.R.,V Herrmann et al. 1994 Microflora on explanted silicon rubber voice prostheses: Taxonomy, hydrophobicity and electrophoretic mobility. *Journal of Applied Bacteriology*;76:521-528.

[2] Mahiea H. F., Van Saene H. K. F., Rosingh H. J., Schutte H K., 1986.*Candida* vegetation on silicone voice prosthesis. *Arch. Otolaryngeal Head Neck Surgery*.; 112: 321-325.

[3] C. P. Girish Kumar, Thangam Menon, 2006.Biofilm production by clinical isolates of *Candida* species. *Medical Mycology*, 44:99-101.

[4] H.C.Van der Mei et al, 2000. Effect of probiotic bacteria on prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses in vitro. *J Med Microbiology*, 49: 713-718.

[5] Sunit C. Singh and A. Baranwa, 2008. Probiotic use in the critically ill. *Indian Journal of Pediatrics*, 6:621-627.

[6] F. Perezous, Catherine M. Flaitz, Millicent E. Goldschmidt, Robert L. Engelmeier, 2005.Colonization of *Candida* species in denture wearers with emphasis on HIV infection: A literature review *The Journal of Prosthetic Dentistry.* Published by Elsevier Inc. 93: 288-293.

[7] Wagner R. D., Pierson C. Warner T. et al, 1997.Biotherapeutic effects of probiotic bacteria on Candidiasis in immunodeficient mice, *Infection Immunity*, 65: 4165-4172.

[8] Velraeds M.C.M, Van der Mei et al., 1996.Physicochemical and biochemical characterization of biosurfactants released by *Lactobacillus* strains. *Biointerfaces*,8:51-61.

[9] Morris D. L,1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science.*, 107:254–255.

[10] M. Al-Fattnai, L.J. Douglas,2006. Biofilm Matrix of *Candida albicans* and *Candida tropicalis* : Chemical composition and role in drug resistance. *J Med Microbiology*, 55:999-1008.

[11] Baielle G. S. and L J Douglas., 1999. *Candida* biofilms and their susceptibility to anti-fungal agents. *Methods of Enzymology*, 310:644-656.

[12] Van Weissenbruch R. , Bouekaert S ,Remoan JP, Nelis HJ , Aerts R, Aibers FWJ. Chemoprophylaxis of fungal deterioration of the Provox Silicone tracheoesophageal prostheses in postlaryngectomy patients.*Ann Otol Rhinol Laryngol* 1997;106:329-337.

[13] Mahieu HF, Van Saene HKF, Rosingh HJ , Schutte HK . *Candida* vegetations on silicone voice prosthesis. *Arch otolaryngol Head Neck Surg*. 1986 .112: 321-325.

[14] Blom ED , Hamaker RC, Tracheoesophageal voice restoration following total laryngectomy.In:Myers E N, Suen JY(eds) *Cancer of the head and neck* ,3 rd edn. Philadelphia Saunders .1196: 839-852.

[15] Isolauri E, Salminen S; 2008 Probiotics: use in allergic disorders: a Nutrition, Allergy, Mucosal Immunology, and Intestinal Microbiota (NAMI) Research Group Report. *J Clin Gastroenterol.*, 42 Suppl 2:S91-6.

[16] Julio Villena, Silvia Racedo, Graciela Agüero, Elena Bru, Marcela Medina and Susana Alvarez. June 2005 *Lactobacillus casei* Improves Resistance to Pneumococcal Respiratory Infection in Malnourished Mice[1] *J. Nutr*., 135:1462-1469,.

[17] Jyotsna Chandra, Pranab K. Mukherjee, 2004. Biofilm formation by fungal pathogen *Candida albicans*: Development, architecture and Drug resistance. *Journal of Bacteriology*, 183:5385-5394.